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14. ABSTRACT- Initially prostate cancer development is driven by the male hormone androgen through the androgen receptor, however, in some patients androgen receptor becomes dysfunctional at late stages of tumorigenesis. Early knowledge of the androgen receptor dysfunctions, what would make prostate tumors refractory to routine androgen ablation therapy, should help in patient stratification for other emerging therapeutic strategies. We proposed a novel approach for evaluating potential dysfunctions of the androgen receptor by measuring the expression of a functionally relevant panel o f genes. This approach is direct and can be easily addressed from human prostate cancer tissues (surgery or diagnostic biopsy specimens) at early stages of the disease. Within the first reporting period we hacve designed, tested and completed the quality control of specific proves and primers evaluating the androgen dose and time kinetics of endogenously expressed PSA/KLK3. PMEPA1, NKX3.1, ODC11, AMD1 and TMPRSS2-ERG genes in VCaP cell culture model. Preliminary evaluation of an initial number of whole-mounted sections of RP specimens with prostate cancer by the Immunohistochemical assessment of AR, ERG , NKX3.1, and PSA proteins indicated reduced expression of ARP genes in a subset of th4e cases. The proposed research we will provide a quantitative incex of AR dysfunction for enhancing disease prognostic characteristics to improve accuracy and to stratify patients for specific therapeutic approaches at early stages of prostate cancer treatment.

15. SUBJECT TERMS- Prostate Cancer, Androgen receptor function, Androgen regulated genes, Therapeutic stratification

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INTRODUCTION

Although most prostate cancers are initially responsive to androgen ablation therapy, they become treatment resistant as tumor cells develop mechanisms to evade the treatment. Multiple mechanisms invoking gain of AR signaling (increased intra-tumoral androgen bio-synthesis, elevated AR function) or loss of AR signaling (activation of AR independent survival pathways) can lead to androgen ablation refractory or Castration Resistant Prostate Cancer (CRPC). Dysfunctions (both gain and loss) of the male hormone receptor, the androgen receptor (AR) mediated signaling play important roles in CaP development or progression (Richter, Srivastava et al. 2007) (Ryan and Tindall 2011). Although AR expression can be detected throughout prostate tumorigenesis, the diagnostic or prognostic utility of monitoring AR levels has been challenging. Studies evaluating the association of AR protein levels with poorly differentiated tumors, higher Gleason score or with decreased PSA recurrence-free survival are inconclusive (Romics, Banfi et al. 2008), (Qiu, Leuschner et al. 2008),(Wako, Kawasaki et al. 2008), (Szabo, Bartok et al. 2009) (Szabo et al 2009). **We reasoned that evaluation of AR dysfunction may be predictive of poor outcome and androgen ablation therapy failure. The proposed approach would significantly impact the patient stratification for new emerging therapeutic strategies.**

Novel insights into the androgen regulated transcriptome have provided by several prostate cancer research laboratories, including ours. These endeavors identified direct transcriptional regulatory targets of AR which have promise in defining the role of AR dysfunctions, as well as, in providing novel, functionally relevant biomarkers and potential therapeutic targets (Xu, Srikantan et al. 2000),(Segawa, Nau et al. 2002), (Velasco, Gillis et al. 2004), (Dehm and Tindall 2006), (Wang, Li et al. 2007) (Richter, Srivastava et al. 2007), (Heemers and Tindall 2009), (Li, Xu et al. 2008) (Li et al 2008), (Wang, Li et al. 2009), (Mostaghel, Geng et al. 2010). Previous, as well as, recent studies revealed androgen regulation of *ERG (TMPRSS2-ERG), KLK3(*PSA*), NKX3.1, PMEPA1, AMD1* and *ODC1* genes in prostate cancer cells (Xu, Shanmugam et al. 2000; Segawa, Nau et al. 2002; Masuda, Werner et al. 2005; Tomlins, Rhodes et al. 2005; Mostaghel, Geng et al. 2010). Decreased expression of androgen regulated genes in association with attenuated response to 5α-reductase inhibition in benign prostate tissues has also been reported (Mostaghel, Geng et al. 2010). The goal of these studies is to provide early warning signs of androgen independence and to predict failure of treatment response. Recent data obtained from prostate cancer cell culture models highlighted a distinct AR regulated transcription program in androgen blockade resistant derivatives of LNCaP cells (Wang, Li et al. 2009). **Although AR can be altered by numerous mechanisms, the net effect of these changes is reflected in defective transcription factor functions of the AR.**

We have reported an overall decrease in androgen regulated gene expression with prostate cancer progression (Sterbis, Gao et al. 2008). Other reports have also noted a signature of attenuated AR function in late stage, especially in metastatic prostate cancer in human specimens (Varambally, Yu et al. 2005), (Hermans, van Marion et al. 2006), (Tomlins, Mehra et al. 2007), (Mendiratta, Mostaghel et al. 2009), as well as in a xenograft models (Hendriksen, Dits et al. 2006). In a recent evaluation of an AR regulated gene panel in selected tumor specimens we reported the association of attenuated AR regulated gene expression with poorly differentiated tumor phenotype at the time of radical prostatectomy and with biochemical recurrence (Dobi, Furusato et al. 2010). **AR can be altered *via* numerous mechanisms, however the net effect of these changes is reflected in defective transcription factor functions of the AR.**

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BODY

To develop readouts for AR function in CaP cells, this DoD/CDMRP grant award focused on the quantitative measurements of AR regulated genes in carefully isolated benign and tumor cells and in prostate tumor tissues.

The hypothesis of this proposal is that the in vivo status of AR function in prostate tumor cells can be precisely defined by measuring the expression of a panel of AR regulated genes (*KLK3(PSA)*, *PMEPA1*, *TMPRSS2-ERG(*ERG), *NKX3.1, AMD1* and *ODC1*). The objective of this proposal is to predict the course of prostate cancer progression by monitoring a panel of AR regulated genes in stratifying patients for treatment modalities.

The findings reported here reflect the progress of the first year of the proposal towards the evaluation of androgen receptor function in prostate cancer prognosis and therapeutic stratification in prostate cancer cells.

Aim #1: To establish the AR regulated gene panel (ARP) as a quantitative measure of *in vivo* AR functional status in prostate cancer at the time of radical prostatectomy.

Tasks to perform:

Task 1: to establish the AR regulated gene panel (ARP) as a quantitative measure of *in vivo* AR functional status in prostate cancer at the time of radical prostatectomy.

Step 1 (Months 1-6):
Institutional Review Board regulatory review and approval processes will occur at two separate institutions: the Walter Reed Army Medical Center (WRNMMC) and the Uniformed Services University of the Heath Sciences (USUHS).
While IRB approval is still ongoing, probes and primers will be designed following the same principles we have been using for TaqMan and will be tested for specificity and sensitivity by using copy number defined dilutions of cDNA clones of ARP.

Step 2 (Months 6-22):
RNA samples for QRT-PCR will be obtained from radical prostatectomy specimens of 110 CaP patients following our established strategy. Total RNA from laser-captured microdissected (LCM) normal and cancer cells from either formalin fixed paraffin embedded (FFPE) or OCT embedded and H&E stained frozen prostate sections of radical prostatectomy specimens (5-10,000 epithelial cells per sample) will be acquired from the CPDR Biospecimen Bank. Total RNA will be quantified by using RiboGreen fluorometric method. The total RNA isolated from the paired tumor and normal LCM epithelium specimens will be converted to cDNA. The expression levels of ARP genes (*PSA/KLK3, PMEPA1, NKX3.1, ODC1, AMD1* and *TMPRSS2-ERG*) will be determined in the matched tumor and normal prostate tissue-derived cDNA samples by real time QRT-PCR (TaqMan). The expression of *GAPDH* will be simultaneously analyzed as endogenous control.

<u>Step 3</u> (Months 23-24):
Gene AR panel expression data will be analyzed by informatic and statistical methods for positive or negative correlation with aggressiveness of prostate cancer, as defined by Gleason grade, pathological stage, biochemical recurrence and for feasibility of gene expression measurement in a clinical assay (specificity, sensitivity and reproducibility). Cumulative index will be used for quantitative definition of AR function (ARF index) towards determine the stratification power of AR gene panel at the time of radical prostatectomy. This index will be incorporated into nomograms modeling time-to-event data, including prediction of disease progression, combined with established clinical and pathological characteristics that predict this study endpoint.

Accomplished

<u>Step 1</u> (Months 1-6):
- Institutional Review Board regulatory review and approval was obtained from two separate institutions: the Walter Reed Army Medical Center (WRAMC) and the Uniformed Services University of the Heath Sciences (USUHS).
- The primers and probes were designed for TaqMan assay of *PSA/KLK3, PMEPA1, NKX3.1, ODC1, AMD1* and *TMPRSS2-ERG* genes, quality control has been completed and probes has been tested for specificity and sensitivity.

Novel Findings

To monitor the functional status of androgen receptor in prostate tumors a panel of six androgen inducible genes were selected. This panel includes tissue (*KLK3(*PSA)), AR stability regulator (*PMEPA1*), transcription factor (*NKX3.1*), polyamine biosynthesis (*ODC1, AMD1*) and oncogene (*TMPRSS2-ERG)* protein coding genes. Selected genes are either direct targets of AR or are tightly regulated by AR and have demonstrated prostate associated expression encompassing major biological functions regulated by AR in the human prostate. The primers and probes have been obtained, and tested by using cDNA from VCaP prostate cancer cell line endogenously expressing all target genes, *PMEPA1* (Locus ID: GXL_128240), *TMPRSS2-ERG* fusion A (Locus ID: GXL_39091), *KLK3(PSA)* (LocusID: GXL_32002), *NKX3.1* (LocusID: GXL_29827), *AMD1* (Locus ID: GLX_261249, and *ODC* (Locus ID: GXL_75806). PCR amplicons were isolated and target regions were confirmed by DNA sequencing.

To assess the performance of primers and probes assessing androgen dose and time kinetic response of target genes, VCaP cells were grown in androgen depleted conditions for four days. To induce androgen regulated genes, synthetic androgen (R1881) was added to the cell cultures at 0, 0.1, 1.0 and 10nM concentrations and cells were harvested at 0, 12, 24 and 48h time points. Cell morphology in response to R1881 treatment was monitored by microscopy (Figure 1.).
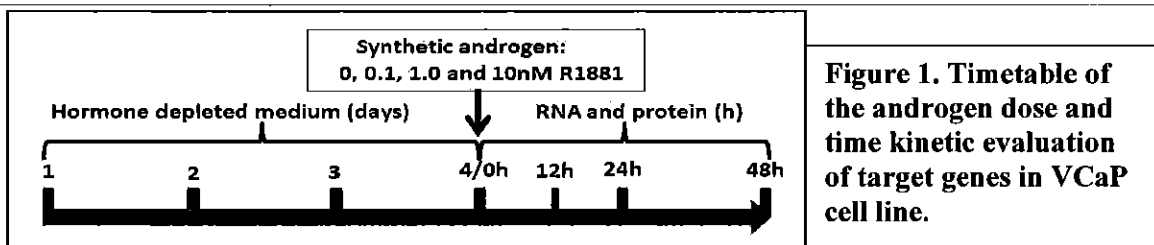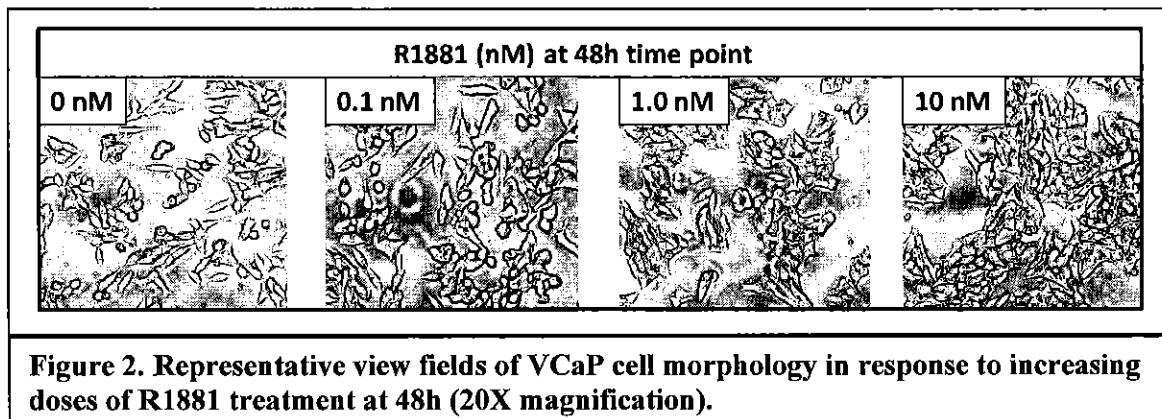

Figure 1. Timetable of the androgen dose and time kinetic evaluation of target genes in VCaP cell line.

To monitor changes in cell morphology in response to R1881 induction, cell were assessed at 0, 12, 24 and 48h time points. The observed cell morphology was consistent with the expected time and dose kinetic response of VCaP cells to R1881 treatment (Figure 2.).

Figure 2. Representative view fields of VCaP cell morphology in response to increasing doses of R1881 treatment at 48h (20X magnification).

To evaluate protein products of three of the target genes (PSA, NKX3.1 and ERG) that was previously shown robust response to androgen induction in hormone responsive prostate cancer cell culture models (Xu et al., 2001; Segawa et al., 2002; Tomlins et al., 2005), VCaP cells were harvested and cell lysates were prepared at 0, 12, 24 and 48h time points in the 0, 0.1, 1.0 and 10 nM R1881 treatment groups. Expression of PSA, NKX3.1 and ERG proteins were assessed by immunoblot assays. As
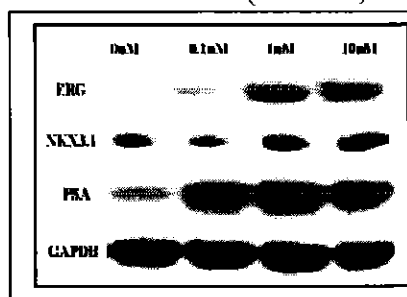

Figure 3. Expression of ERG, NKX3.1 and PSA protein in response to increasing doses of R1881 at 48h time point in VCaP cells. GAPDH was used as the loading control in the immunoblot assay.

expected, the experiment revealed androgen hormone dose dependent expression of the assayed proteins (Figure 3.).

For the evaluation of ARP target gene expressions RNA was isolated from VCaP cells at 0, 12, 24 and 48h in the 0, 0.1, 1.0 and 10 nM R1881 treatment groups. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in triplicates and one control reaction without RT were performed for each RNA sample by using primer and probe sets for *KLK3*(PSA), *TMPRSS2-ERG*(fusion junction "A" (Tomlins *et al.*, 2005)), *NKX3.1*, *PMEPA1*, *ODC1* and *AMD1* genes and expression values were expressed relative to *GAPDH*. Expression results were calculated from the average C_T (threshold cycle) values of triplicates (Figure 4.).
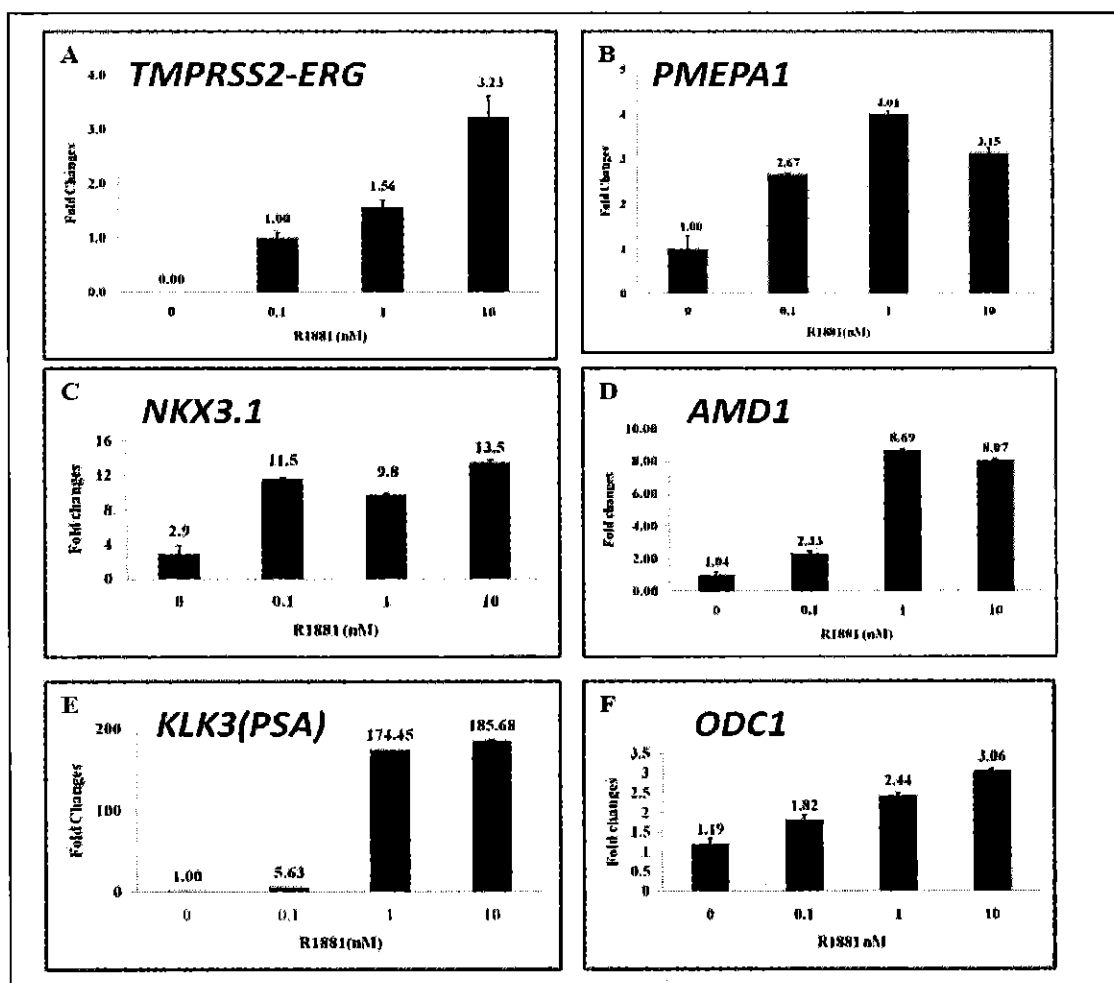
Figure 4. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) of *KLK3*(PSA), *TMPRSS2-ERG*(ERG), *NKX3.1*, *PMEPA1*, *ODC1* and *AMD1* genes in VCaP cells demonstrate the activation of ARP genes in response to increasing doses of R1881 at 48h time point. Experiments were performed in triplicates and expression levels relative to *GAPDH* are shown as fold changes.

Specific primers and probes were designed for the qRT-PCR analyses of *PSA/KLK3*, *PMEPA1*, *NKX3.1*, *ODC1*, *AMD1* and *TMPRSS2-ERG* genes. Quality control of primer and probe sets has been completed. Kinetic and androgen dose response of endogenously expressed levels of ARP genes in VCaP cells indicate the sensitivity of detection. Sequence analyses of target gene amplicons confirmed the specificity of detecting ARP genes.

Aim #2: to define the utility of ARP proteins in monitoring the AR function.

Tasks to perform:

Task 2: Define the utility of ARP proteins in monitoring the AR function.

Step 1 (Months 12-30):
IHC will be set up and optimized with antibodies against ARP gene products. Whole-mounted sections of RP specimens with prostate cancer will be assayed in a cohort of over 110 patients by immunohistochemistry. The staining intensities will be determined according to percent of cells positive. The intensity will be scored and a combination of measurements will be calculated by multiplying the percent of positive cells with the degree of intensity, which will result in an IHC intensity score. The sum of staining intensity scores will be expressed as the cumulative IHC staining index of AR regulated gene panel.

Step 2 (Months 12-30):
To establish concordance between the expression of ARP mRNA and proteins branched-chain DNA (b-DNA) signal amplification method will be used. Adjacent 4 μm-thick section will be selected from each of the FFPE whole-mount prostate samples.

Step 3 (Months 31-36):
Incorporate the AR gene panel cumulative indexes into predictive nomograms for prostate cancer progression defining predictive power and utility for patient stratification.
Translational products of the AR regulated gene panel will be assessed in formalin fixed paraffin embedded (FFPE) tissues by evaluating 110 whole-mounted radical prostatectomy specimens. To address the concordance between mRNA and protein expression adjacent sections will be analyzed by the recently developed bDNA technology. From the IHC staining of products of the ARP will be summarized in a cumulative index for patient stratification. IHC scores and a cumulative pathology scores will be established for the tumor foci in the sections. Cumulative IHC score will be evaluated alone and by combining with nomograms modeling time-to-event data incorporating the biochemical recurrence within eight years of follow up. Post-operative predictive value of existing, validated nomograms will be assessed by using the patient cohort. The IHC-derived ARP values, as a single cumulative index, will be incorporated into validated nomograms (Kattan) modeling time-to-event data, including prediction of CaP progression, combined with established clinical and pathological characteristics that predict this study endpoint. The concordance index, C, will be used to assess the improvement in model fit upon inclusion of AR function index.

Accomplished

Step 1 (Months 6-12):
- The Whole-mounted sections of RP specimens with prostate cancer have been identified in a cohort of over 110 patients for immunohistochemistry.

Step 2 (Months 9-12):
- We have focused our initial Immunohistochemical assessment of ARP genes on the patient cohort that we have previously defined for ARP gene defects in microarray-based gene expression studies (Dobi et al., 2010). IHC has been completed for 40 cases with anti- ERG, PSA, NKX3.1 and AR and antibodies. The staining intensities have been determined according to percent of cells positive. The intensity was

scored and a combination of measurements was calculated by multiplying the percent of positive cells with the degree of intensity (IHC intensity score).

Novel Findings

Radical prostatectomy specimens were selected from 40 cases that were previously fixed in 10% buffered formalin and embedded as whole mounts in paraffin. Each prostate was sectioned at 0.22 cm intervals in a transverse plane perpendicular to the long axis of the posterior surface of the prostate and completely embedded as whole mounts. IHC staining on adjacent four-micron sections of the whole-mounted blocks were performed by using mouse monoclonal anti-ERG antibody developed by our laboratory (CPDR ERG-MAb, (Furusato, Tan et al. 2010), (Mohamed, Tan et al. 2010), (Miettinen, Wang et al. 2011) (Braun, Goltz et al. 2011) (Rosen, Sesterhenn et al. 2012), rabbit polyclonal anti-PSA antibody (DAKO, A056201-2), rabbit polyclonal anti-NKX3.1 antibody (a kind gift from Dr. Charles Bieberich, UMBC) and mouse monoclonal anti-AR antibody (SantaCruz, sc-7305) (Figure 5.). Slides were incubated with these antibodies and were counterstained with hematoxylin. The protein expression was assessed based on both the amount and intensity of immunopositive cells. Intensities were scored from 0 to 2 as follows: 0 as non-stained, 1 as weak, and 2 as strong. The percentage of positively stained area was estimated and scored from 1 to 4 as follows: less than 25% positive area as score 1, 25-50% score 2, 51-75% score 3, and more than 75% positive area score 4. The final score was determined after multiplying the intensity score and percentage of positively stained area in the respective lesions.
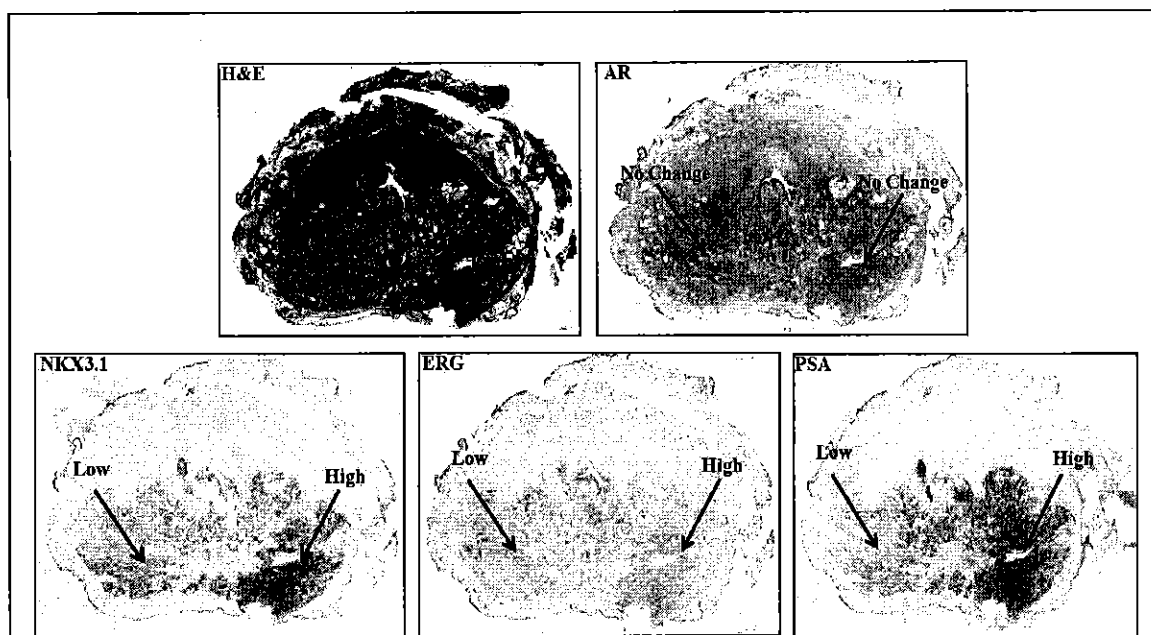

Figure 5. Initial assessment of whole-mount prostate sections indicates attenuated expression of ARP genes in a sub-set of prostate tumors. On the upper left panel two tumor foci (T1 and T2) are shown by H&E. The upper right panel shows equal AR immunostaining is in both tumor foci (no change). Lower panels show reduced immunoreactivity of NKX3.1, ERG and PSA in the left tumor focus (Low), in contrast to the tumor focus shown at the right side (High) of the whole-mount prostate section.

Initial assessment of AR dysfunction by the immunohistochemical evaluation of ARP genes in 40 whole mount prostate cancer specimens indicate that in a sub-set of cases tumor foci can be identified with reduced expression of ARP genes. Although, these data are preliminary, if consistently observed in further experiments it will support the central hypothesis of the proposal that AR function in prostate tumor cells can be defined by measuring the expression of a panel of AR regulated genes.

KEY RESEARCH ACCOMPLISHMENTS

Aim #1: To establish the AR regulated gene panel (ARP) as a quantitative measure of *in vivo* AR functional status in prostate cancer at the time of radical prostatectomy.

- Towards the successful accomplishment of the proposal a key step was to obtain Institutional Review Board approval from the Walter Reed National Military Medical Center (WRNMMC) and from the Uniformed Services University of the Heath Sciences (USUHS).

- The primers and probes were designed for qRT-PCR assays for assessing the expression of *PSA/KLK3, PMEPA1, NKX3.1, ODC1, AMD1* and *TMPRSS2-ERG* genes. The quality control of primers and probes for the detection of endogenously expressed ARP genes has been completed, probes specificities and sensitivities were confirmed.

Aim #2: to define the utility of ARP proteins in monitoring the AR function.

- The Whole-mounted sections of RP specimens with prostate cancer have been identified in a cohort of 110 patients for the Immunohistochemical assessment of ARP proteins.

- IHC has been completed for 40 cases for ERG, PSA, NKX3.1 and AR. The staining intensities have been determined and intensity was scored and a combination of measurements was calculated. Initial assessment of ARP genes by IHC shows tumor foci with reduced expression of ARP genes in a sub-set of cases.

REPORTABLE OUTCOMES

<u>Publications</u>

None

<u>Poster presentations</u>

American Urological Association (AUA) Annual Meeting, Atlanta, GA, May 19 - 23, 2012.

Shyh-Han Tan, Christian Ruiz, Martin Braun, Veit Scheble, T Zellweger, CA Rentsch, A Bachmann, Albert Dobi, Sven Perner, Isabell Sesterhenn, Shiv Srivastava, L Bubendorf: **Comprehensive analysis of the TMPRSS2-ERG translocation during prostate cancer progression.**

Press Releases

None

Funding Applied for Based on Work Supported by this Award

None

Employment or research opportunities applied for and/or received based on experience/training supported by this award

This award supports the employment and post-doctoral training of Shashwat Sharad, PhD. He has completed the qRT-PCR analysis of *PSA/KLK3, PMEPA1, NKX3.1, ODC1, AMD1* and *TMPRSS2-ERG* genes in VCaP cells. His research results revealed that the qRT-PCR primers and probes employed in the proposal are suitable for the detection of endogenous levels of ARP genes.

The Research Assistant position of this award, in part supports the salary of Ms. Wei Huang, MS., a full-time employee of CPDR. She has experience in key techniques pertinent to this proposal. Ms. Huang prepared the cell culture models, protein analyses and assisted in the completion of all qRT-PCR experiments. Also, she has completed the DNA sequence confirmation of PCR ARP amplicons.

List of Personnel (not salaries) receiving pay from the research effort

PI: Albert Dobi, PhD.
Co-I: Gyorgy Petrovics, PhD.
Biostatistician: Yongmei Chen, MD, MPH.
Post-doctoral Fellow: Shashwat Sharad, PhD.
Research Assistant: Wei Huang, MS.

CONCLUSIONS

The central hypothesis of the proposal that AR function in prostate tumor cells can be defined by measuring the expression of a panel of AR regulated genes. Towards defining the expression levels of AR regulated genes in prostate cancer we have designed, tested and completed the quality control of specific probes and primers evaluating the androgen dose and time kinetics of endogenously expressed *PSA/KLK3, PMEPA1, NKX3.1, ODC1, AMD1* and *TMPRSS2-ERG* genes in VCaP cell culture model.

Initial evaluation of whole-mounted sections of 40 radical prostatectomy specimens with prostate cancer by the Immunohistochemical assessment of AR, ERG, NKX3.1 and PSA proteins indicated reduced expression of ARP genes in a subset of the cases.

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APPENDIX

Comprehensive analysis of the *TMPRSS2-ERG* translocation during prostate cancer progression

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Introduction:
Approximately half of the diagnosed prostate carcinomas (PC) are characterized by a chromosomal rearrangement fusing the androgen regulated gene *TMPRSS2* to the oncogenic *ETS* transcription factor *ERG*. Aim of this study was to comprehensively analyze the impact of this translocation on the expression of the *ERG* gene in hormone-naïve (untreated) and castration-resistant prostate cancers and to define the influence of AR protein expression and genomic amplification in this context.

Methods:
We constructed a tissue microarray (TMA) containing 915 tissue cores from 107 hormone-naïve PC and from 101 castration-resistant (CR) PC. In addition, we included 56 specimens from distant metastases. We analyzed the *TMPRSS2-ERG* translocation status by fluorescence in-situ hybridization and the expression profiles of ERG, AR and the proliferation marker Ki67 by immunohistochemistry.

Results:
Nearly half of the analyzed PC tissue specimens (untreated: 38%, castration-resistant: 46%) harbored a *TMPRSS2-ERG* gene fusion. Untreated PC with positive translocation status showed increased tumor cell proliferation ($p < 0.05$). As expected, *TMPRSS2-ERG* gene fusion was strongly associated with increased ERG protein expression in untreated, as well as in castration-resistant PC (both $p < 0.0001$). However, we detected a subgroup (26%) of CR PCs with the gene fusion, but without detectable ERG protein expression. This subgroup showed significantly lower levels of AR protein expression and of the androgen regulated serum PSA (both $p < 0.05$).

Conclusions:
This comprehensive study comparing the *TMPRSS2-ERG* gene and ERG protein expression status in a large cohort of hormone-naïve and CR PC, identifies a subgroup of translocated CR PCs without ERG protein expression. **Our results suggest that this subgroup may represent CR PCs with a dispensed AR pathway.** Further analyses may help to explain if these tumors represent a distinct subgroup of AR independent CR PCs.